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(54) Title: VIRULENCE OF STREPTOCOCCI

(57) Abstract: The invention relates to the field of diagnosis of and vaccination against Streptococcal infections and to the detection of virulence markers of Streptococci. The invention provides a method for modulating virulence of a Streptococcus comprising modifying a genomic fragment of said Streptococcus wherein said genomic fragment comprises at least a functional part of a fragment identifiable by hybridisation in Streptococcus suis to a nucleic acid or fragment thereof as shown in figure 5.

The invention relates to the field of diagnosis of and vaccination against Streptococcal infections and to the detection of virulence markers of Streptococci.

Streptococcus species, of which there are a large variety causing infections in domestic animals and man, are often grouped according to Lancefield's groups.

5 Typing according to Lancefield occurs on the basis of serological determinants or antigens that are among others present in the capsule of the bacterium and allows for only an approximate determination, often bacteria from a different group show cross-reactivity with each other, while other Streptococci can not be assigned a group-determinant at all. Within groups, further differentiation is often possible on the

10 basis of serotyping; these serotypes further contribute to the large antigenic variability of Streptococci, a fact that creates an array of difficulties within diagnosis of and vaccination against Streptococcal infections.

Lancefield group A *Streptococcus* (GAS, *Streptococcus pyogenes*), are common with children, causing nasopharyngeal infections and complications thereof. Among

15 animals, especially cattle are susceptible to GAS, whereby often mastitis is found.

Lancefield group B *Streptococcus* (GBS) are most often seen with cattle, causing mastitis, however, human infants are susceptible as well, often with fatal consequences. Group B streptococci (GBS) constitute a major cause of bacterial sepsis and meningitis among human neonates born in the United States and Western

20 Europe and are emerging as significant neonatal pathogens in developing countries as well.

Lancefield group C infections, such as those with *S. equi, S. zooepidemicus, S. dysgalactiae*, and others are mainly seen with horse, cattle and pigs, but can also cross the species barrier to humans.

25 Lancefield group D (*S. bovis*) infections are found with all mammals and some birds, sometimes resulting in endocarditis or septicaemia.

Lancefield groups E, G, L, P, U and V (*S. porcinus, S, canis, S. dysgalactiae*) are found with various hosts, causing neonatal infections, nasopharyngeal infections or mastitis.

Within Lancefield groups R, S, and T, (and with ungrouped types) *S. suis* is found, an important cause of meningitis, septicemia, arthritis and sudden death in young pigs. Incidentally, it can also cause meningitis in man.

Ungrouped *Streptoccus species*, such as *S. mutans*, causing carries with

5 humans, *S, uberis*, causing mastitis with cattle, and *S. pneumonia*, causing major infections in humans, and *Enterococcus faecilalis* and *E. faecium*, further contributed to the large group of Streptococci. *Streptococcus pneumoniae* (the pneumococcus) is a human pathogen causing invasive diseases, such as pneumonia, bacteraemia, and meningitis.

10 Little is known about the pathogenesis of the disease caused by Streptococci. Various cellular components, such as muramidase-released protein (MRP) extracellular factor (EF) and cell-membrane associated proteins, fimbriae, haemagglutinins, and haemolysin have been suggested as virulence factors. However, the precise role of these protein components in the pathogenesis of the disease

15 remains unclear. It is however, well known and generally accepted that the polysaccharidic capsule of various Streptococci and other gram-positive bacteria plays an important role in pathogenesis. The capsule enables these micro-organisms to resist phagocytosis and is therefore regarded as an important virulence factor or marker.

20 In particular, *Streptococcus suis* is an important cause of meningitis, septicemia, arthritis and sudden death in young pigs (6, 31). It can also cause meningitis in man (2). Attempts to control the disease are still hampered by the lack of sufficient knowledge about the pathogenesis of the disease and the lack of effective vaccines and sensitive diagnostic methods.

25 So far, 35 serotypes of S. *suis* are described (8, 9, 10). Virulence of *S. suis* can differ within and among serotypes (29, 30, 32, 33). Worldwide *S. suis* serotype 2 is the most frequently isolated serotype. Within *S. suis* serotype 2, pathogenic, weak-pathogenic and non-pathogenic strains can be found (32, 33). The pathogenic strains cause severe clinical signs of disease in pigs and large numbers of bacteria can be

30 reisolated from the central nervous system (CNS) and the joints after experimental infection (32, 33). The weak-pathogenic strains cause only mild clinical signs of disease and only infrequently bacteria can be reisolated from the CNS and the joints

after experimental infection (32, 33). The non-pathogenic strains are completely avirulent in young pigs after experimental infection (32, 33).

The 136-kDa muramidase-related protein (MRP) and the 110-kDa extracellular factor (EF) are generally considered as important virulence markers for *S. suis*
5   serotype 2 strains isolated in Europe and the United States (3, 7, 16, 21, 28, 35). However, differences in virulence between pathogenic, weak-pathogenic and non-pathogenic strains cannot exclusively be explained by differences in their MRP and EF expression patterns (26). In addition, it is known that the capsule of *Streptococcus suis* serotype 2 is an important virulence factor (23). However, since both pathogenic,
10   weak-pathogenic and non-pathogenic strains seemed to be fully encapsulated after growth *in vitro* and *in vivo*, it is not likely that the level of encapsulation of these fully encapsulated strains is associated with their difference in virulence.

The invention provides a method for modulating virulence of a *Streptococcus*
15   comprising modifying a genomic fragment of said *Streptococcus* wherein said genomic fragment comprises at least a functional part of a fragment identifiable by hybridisation in *Streptococcus suis* to a nucleic acid or fragment thereof as shown in figure 5. To get insight in the differences between pathogenic, weak-pathogenic and non-pathogenic strains, that determined their difference in virulence, the invention
20   provides an *in vivo* complementation system whereby virulence can be modified by modifying said fragment. For example, within *S. suis* serotype 2 pathogenic, weak-pathogenic and non-pathogenic strains can be found. We introduced a genomic library of a pathogenic strain into a weak-pathogenic strain. After infection of the library into young piglets pathogenic transformants were selected. One specific
25   transformant, containing a 3-kb fragment of the pathogenic strain, V10, appeared to be dominantly enriched in diseased pigs. The observed enrichment was not tissue specific. The selected fragment, when introduced into two different weak-pathogenic strains, increased the virulence of these strains considerably. In particular, the fragment herein identified as ORF 2, or functional fragments thereof is an important
30   virulence factor. In contrast, introduction of the corresponding fragment of a weak-pathogenic strain had only minor effects on virulence. Thereby, the invention also provides a method for assaying virulence of a *Streptococcus* comprising assaying a genomic fragment of said *Streptococcus* wherein said genomic fragment comprises at

least a functional part of a fragment identifiable by hybridisation in *Streptococcus suis* to a nucleic acid or fragment thereof as shown in figure 5, in particular the ORF 2 fragment. Nucleotide sequence analysis of the selected fragment of the pathogenic strain revealed the presence of two potential open reading frames both of which were found to be mutated in the corresponding fragment of the weak-pathogenic strain. It was previously shown by ribotyping and random amplified polymorphic DNA analysis (RAPD) assays, that pathogenic and weak-pathogenic strains of *S. suis* serotype 2 are genetically closely related, whereas non-pathogenic strains showed a high degree of genetic heterogeneity (5, 24). We constructed a genomic library of the pathogenic *S. suis* strain 10 in plasmids and introduced the plasmid library into the weak-pathogenic reference strain of *S. suis* serotype 2, strain S735 (34). Pigs were inoculated intravenously with the recombinants and bacteria were recovered from the CNS and the joints of diseased pigs. The re-isolated bacteria were subsequently analyzed for their plasmid content and their virulence. With this approach we identified a DNA fragment of a pathogenic serotype 2 strain that transformed weak-pathogenic strains into highly pathogenic strains. This fragment as provided herein comprises a genetic determinant important for virulence. Said fragment is in other Streptococci identifiable by for example hybridisation experiments such as Northern or Southern blotting or by amplification experiments such as PCR using primers and/or probes derivable from a nucleic acid as provided herein.

With the fragment, and parts thereof, such as the open reading frames identified in figure 3, a virulence marker is provided herein. Said marker is associated with an isolated and/or recombinant nucleic acid as provided herein and derivable from *Streptococcus* and identifiable by hybridisation in *Streptococcus* (preferably *S. suis*) to a nucleic acid or fragment thereof as shown in figure 5.

The invention also provides a vector comprising a nucleic acid according to the invention, and a host cell comprising a nucleic acid or a vector according to the invention. Such a host cell preferably comprises an easily modifiable organism such as E. coli, however, other host cells, such as recombinant *Streptococcus* (preferably derived from one of the grouped or ungrouped *Streptococci* as identified herein above) comprising a vector or nucleic acid according to the invention are herein also provided. In particular, recombinant *Streptococcus* as provided herein is useful for inclusion in a vaccine.

Furthermore, the invention provides a vaccine comprising a nucleic acid or a vector or a host cell according to the invention, and use of such a vaccine in the prevention and/or treatment of Streptococcal infections.

Also provided is a protein or fragment thereof encoded by a nucleic acid according to the invention, such as a protein encoded by ORF 2 or ORF 3 as disclosed herein or functional, i.e. antigenic fragment thereof. The invention also provides an antibody directed against a protein or fragment thereof according to the invention and an antigen reactive with such an antibody, for example comprising a protein or fragment. Such a protein or fragment thereof need not be obtained by recombinant means only, synthesis of peptides, according to their amino acid sequence, is equally well possible. Such antigens and antibodies as provided herein can be used in a diagnostic test comprising an antibody according to the invention, or within a vaccine or diagnostic test comprising an antigen according to the invention. Such vaccines and diagnostic tests can be used in the field of diagnosis of and vaccination against Streptococcal infections and for the detection of virulence markers of Streptococci.

Detailed description

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. suis* strains were grown in Todd-Hewitt broth (code CM189, Oxoid), and plated on Columbia agar blood base (code CM331, Oxoid) containing 6% (v/v) horse blood. If required, antibiotics were added at the following concentrations: erythromycin, 1 µg/ml. *E.coli* strains were grown in Luria broth (18) and plated on Luria broth containing 1.5% (w/v) agar. If required, 200 µg/ml of erythromycin was added.

pCOM1. pCOM1 (Fig. 1) is based on the replication functions of pWVO1 (13). Moreover the vector contained the erythromycin-resistance gene of pE194 (11) preceded by the promoter region of the *mrp* gene (25) as well as the *SacI-PstI* part of the multiple cloning site of pKUN19 (14). As the result pCOM1 contained a unique *Bam*HI site (Fig. 1).

Construction of genomic *S. suis* library in pCOM1. *Sau*3AI partial digests of the DNA of the pathogenic *S. suis* serotype 2 strain 10 were size fractionated (> 3 kb) by

precipitation with 4.6% of PEG 6000 (BDH Chemicals, 19). The fragments were ligated to *Bam*HI digested pCOM1 and the ligation mixtures were transformed to *E. coli* XL2-blue cells. Erythromycin-resistant colonies were selected. About 17000 independent *E. coli* clones were obtained. Analysis of 55 of the transformants showed

5   that 64% contained an insert > 3 kb. From the pool of *E. coli* transformants plasmid DNA was isolated, which was subsequently used for the electrotransformation of the weak-pathogenic *S. suis* strain S735 (27). This resulted in approximately 30.000 independent *S. suis* transformants. The *S. suis* library was designated S735(pCOM-L). The transformants were pooled and stored at -80°C.

10   DNA techniques. Routine DNA manipulations were performed as described by Sambrook et al. (22). DNA sequences were determined on a 373A DNA Sequencing System (Applied Biosystems, Warrington, GB). Samples were prepared by use of an ABI/PRISM dye terminator cycle sequencing ready reaction kit (Applied Biosystems). Custom-made sequencing primers were purchased from Life Technologies.

15   Sequencing data were assembled and analyzed using the McMollyTetra software package. The BLAST program was used to search for protein sequences homologous to the deduced amino acid sequences.

          For PCR reaction mixtures (50 μl) the PCR Expand High Fidelity system (Boehringer, Mannheim, Germany) was used as described by the supplier. DNA

20   amplification was carried out in a Perkin Elmer 9600 thermal cycler and the program consisted of an incubation for 2 min at 95°C, 10 cycles of 20 sec at 95°C, 1 min at 60°C and 4 min at 68°C, 30 cycles of 20 sec at 95°C, 1 min at 60°C and 4 min, extended with 20 sec for each cycle, at 68°C and 10 min at 72°C.

          Southern blotting and hybridization. Chromosomal DNA was isolated as

25   described by Sambrook et al. (22). DNA fragments were separated on 0.8% agarose gels and transferred to Gene-Screen Plus membranes (NEN) as described by Sambrook et al. (22). DNA probes were labeled with [(α-^{32}P]dCTP (3000 Ci mmol^{-1}; Amersham) by use of a random primed labeling kit (Boehringer). The DNA on the blots was hybridized at 65°C with the appropriate DNA probes as recommended by

30   the supplier of the Gene Screen Plus membranes. After hybridization, the membranes were washed twice with a solution of 40 mM sodium phosphate, pH 7.2, 1 mM EDTA, 5% SDS for 30 min at 65°C and twice with a solution of 40 mM sodium phosphate, pH 7.2, 1 mM EDTA, 1% SDS for 30 min at 65°C.

Construction of pCOM-V10-ORF2 and pCOM-V10-ORF3.

To construct pCOM-V10-ORF2 we used the primers 5'-CGAGCTCGGAAGAATTGGTTATTGCGCGTG-3' and 5'-CGGGATCCCGGGGGATGACCTGTTGCTTG-3', in a PCR reaction on chromosomal

5 DNA of *S. suis* strain 10 to amplify the ORF 2 encoding region. The resulting fragment was purified, digested with *Sac*I and *Bam*HI and cloned into *Sac*I and *Bam*HI-digested pCOM1. To construct pCOM-V10-ORF3 we used the primers 5'-TCCCCCGGGGGACAAGCAACGGGTCATCCCC-3' and 5'-CGGGATCCCGGTTGAATGCCCGGCAAAGCG-3' to amplify the ORF 3 encoding

10 region. The resulting fragment was digested with *Sma*I and *Bam*HI and cloned into pKUN19. The resulting plasmid was designated pKUN-ORF3. Because the ORF 2 and ORF 3 encoding regions are most probably co-transcibed, we subsequently amplified the promoter region of ORF 2 by using the primers 5'-CGAGCTCGGAAGAATTGGTTATTGCGCGTG-3' and

15 5'-TCCCCCGGGGGAGTCGTGTGTATTCGACAGCGG-3'. The fragment was digested with *Sac*I and *Sma*I and cloned into *Sac*I and *Sma*I digested pKUN-ORF3. The resulting plasmid was digested with *Sac*I and *Bam*HI, the insert fragment was purified and cloned into *Sac*I and *Bam*HI digested pCOMI. This resulted in pCOM-V10-ORF3.

20 Experimental infections. Germfree pigs, crossbreeds of Great Yorkshire and Dutch landrace, were obtained from sows by caesarian sections. The surgery was performed in sterile flexible film isolators. Pigs were allotted to groups, each consisting of 4 or 5 pigs, and were housed in sterile stainless steel incubators. Housing conditions and feeding regimes were as described before (30, 33). One week

25 old pigs were inoculated intravenously with *S. suis* strains as described before (30, 33). Pigs received erythromycin twice a day orally (Erythromycin stearate, Abbott B.V., Amstelveen, The Netherlands, 40 mg/kg body weight). Two hours after the infection the pigs were treated with erythromycin for the first time. Pigs were monitored twice a day for clinical signs of disease, such as fever, nervous signs and

30 lameness. Blood samples were collected three times a week from each pig. White blood cells were counted with a cell counter. To monitor infection with *S. suis* we collected swabs of nasopharynx and feces daily. The swabs were plated directly onto Columbia agar containing 6% horse blood. After the pigs were killed, they were

examined for pathological changes. Moreover, tissue specimens were collected from the central nervous system, serosae, joints, lungs, liver, kidney, spleen, heart and tonsils. The tissues were homogenized in the presence of Todd-Hewitt medium by using an Ultra-Turrax tissuemizer (Omni International, Waterbury, USA),

5   centrifuged for 5 min at 3000 rpm and the supernatants were frozen at -80° C in the presence of 15% glycerol.

RESULTS
10

Complementation system. A genomic library of the pathogenic S. suis strain 10 was constructed into the weak-pathogenic strain S735 as described in Materials and Methods. The plasmid pCOM1 allowed the insertion of large DNA fragments into the unique BamHI site (Fig. 1). The plasmid carries the origin of replication of pWVO1,

15   which functions in E. coli as well as in S. suis (27). This allowed the construction of a DNA library in E. coli first. Plasmid DNA, isolated from the pool of E. coli transformants, was subsequently electrotransformed into S. suis strain S735. 30.000 individual S. suis clones were obtained. As determined by analysis of 24 randomly selected transformants, more than 30% of the S735(pCOM-L) transformants

20   contained an insert > 3kb.

Selection of genomic fragments associated with virulence. To select for genetic determinants of the pathogenic S. suis strain 10 that could increase the virulence of the weak-pathogenic strain S735, pigs were inoculated with the S. suis library S735(pCOM-L). We used a dose of either 10^7 or 10^8 cfu, and the pigs were treated

25   with erythromycin as described in Materials and Methods. All pigs showed specific S. suis symptoms (Table 2A) three to seven days after the infection and except for one, all pigs died during the course of the experiment. From five of the pigs bacteria could be reisolated from the CNS and from two other pigs bacteria were isolated from the joints (Table 2A). In previous experiments, in which pigs were inoculated with weak-

30   pathogenic strains, specific S. suis symptoms were observed with a very low frequency (33, 34). In addition, from those pigs bacteria could never be re-isolated from the CNS nor from the joints. Therefore, these data indicated that compared to virulence of strain S735, bacteria isolated from pigs inoculated with the S. suis

library S735(pCOM-L) are more virulent due to the presence of a DNA fragment of the pathogenic strain 10. The plasmid content of 90 randomly selected clones isolated from the CNS or the joints of the seven diseased pigs was analyzed by PCR and restriction analysis. The results showed that 88 of the 90 clones analyzed (19 of which

5   are shown in Fig. 2) contained an insert of about 3 kb and had identical restriction patterns. Moreover, the inserts of 10 randomly selected clones, having identical restriction patterns, also showed identical DNA sequences (results not shown). Plasmid DNA of 10 randomly selected clones from the original S735(pCOM-L) library showed 10 different restriction patterns (Fig. 2). These data suggest that one specific

10   clone, which was designated S735(pCOM-V10), was greatly enriched in seven different pigs. Moreover, this particular clone was isolated from the CNS as well as from the joints of the various pigs, indicating that the observed enrichment was not tissue specific.

       Virulence-associated properties of the selected fragment V10. To further

15   analyze the virulence properties of strain S735(pCOM-V10), pigs were inoculated intravenously with 10^6 cfu of strain S735(pCOM1) or strain S735(pCOM-V10). The results (Table 2B) clearly show that, compared to the virulence of strain S735(pCOM1), the virulence of strain S735(pCOM-V10) was greatly enhanced. All pigs inoculated with strain S735(pCOM-V10) showed specific *S. suis* symptoms and

20   died within one day after infection. In contrast, except for one, none of the pigs inoculated with the control strain S735(pCOM1) showed specific clinical symptoms and these pigs survived until the end of the experiment (15 days after infection). These data proved that introduction of fragment V10 of strain 10 into S735 transformed the weak-pathogenic strain S735 into a highly pathogenic strain. This

25   strongly suggests that the protein(s) encoded by V10 are important virulence determinants and play an important role in the pathogenesis of *S. suis* serotype 2 infections in pigs.

       To find out whether the observed increase of the fragment V10 on virulence was specific for strain S735, we introduced pCOM1 and pCOM-V10 into another weak-

30   pathogenic strain: strain 24 (33). Subsequently we determined the virulence properties of the strains 24(pCOM1) and 24(pCOM-V10). As shown in Table 2C and 2D, similar effects of V10 on the virulence of strains S735 and 24 were observed. Both strains 24(pCOM-V10) and S735(pCOM-V10) were highly pathogenic for young

piglets, whereas strains 24(pCOM1) and S735(pCOM1) were shown to be only weak-pathogenic (Table 2C and D). This strongly indicates that V10 has a more general ability to transform weak-pathogenic serotype 2 strains into highly pathogenic strains.

5   Because we used a plasmid system for the complementation approach, gene-doses effects cannot be excluded. Plasmid pCOM1 is based on the replication functions of pWVO1. In Gram-positive bacteria the latter plasmid has a copy number between 3 and 6 (13). To find out whether copy effects play a role, we cloned the genomic region of strain S735 homologous to fragment V10 of strain 10 (see below)

10   into plasmid pCOM1. This plasmid was designated pCOM-V735. The virulence of strains S735(pCOM-V735), and 24(pCOM-V735) was subsequently compared to that of S735(pCOM-V10), S735(pCOM1), 24(pCOM-V10) and 24(pCOM1). The results (Table 2C and D) clearly show that, in contrast to pCOM-V10, the plasmid pCOM-V735, did not carry virulence-enhancing activity. Pigs infected with strains

15   S735(pCOM-V10) and 24(pCOM-V10) died within one or two days after infection, whereas most of the pigs infected with strains S735(pCOM-V735), 24(pCOM-V735), S735(pCOM1) and 24(pCOM1) survived until the end of the experiment (17 days after infection). Compared to pigs infected with strains containing pCOM1, pigs infected with strains containing pCOM-V735 developed more general and specific

20   signs of disease, but much less than pigs infected with strains containing pCOM-V10 (Table 2C and D). From these data we concluded that the differences in virulence observed between the strains containing pCOM-V10 and the strains containing pCOM-VS735 are caused by differences between the fragments V10 and V735 (see below). The differences in virulence observed between the strains containing pCOM1

25   and the strains containing pCOM-VS735 may be due to gene-doses effects.

          Sequence analysis of fragments V10 and V735. By using the fragment V10 as a probe a 3.1-kb *PstI-HindIII* fragment of strain S735 (V735) was identified and cloned into pCOM1 (Fig. 3). To analyze the differences between the fragments V10 and V735, we determined the nucleotide sequences of the fragments V10 and V735 and

30   analyzed the sequences for homology to known genes by comparison with the GenBank/EMBL and SWISSPROT databases. The sequence of V10 revealed two complete and two incomplete open reading frames (Fig. 3). ORF 1 (nucleotides 1 to 461) coded for a polypeptide of 153 amino acids. This protein showed homology (49%

identity) to the C-terminal region of acetate kinase of *Clostridium thermocellum* (accession number AF041841) and various other bacterial species (12). ORF 2 (nucleotides 625 to 1327) coded for a protein of 233 amino acids. No significant similarities were found between the predicted amino acid sequence of this protein and proteins present in the datalibraries. ORF 3 (nucleotides 1382 to 2639) coded for a protein of 418 amino acids. This protein showed homology (36% identity) to FolC (folylpolyglutamate synthetase) of *Bacillus subtilis* (15, 17). Compared to the other ORFs, ORF 4 is transcribed in the opposite direction. ORF 4 (nucleotides 2684 to 2972) coded for a polypeptide of 96 amino acids. This polypeptide showed homology (67% identity) to the C-terminal part of PepA (glutamyl-aminopeptidase) of *Lactococcus lactis* (1). Both ORFs 2 and 3 possessed putative initiation codons and ribosome-binding sites. Putative -35 (TGGACA) and -10 (TACAAT) sequences, which may function as promoter sequences, were found preceding ORF 2. ORFs 2 and 3 were separated by 55 nucleotides. In this region no putative promoter sequences could be observed. This could indicate that the ORFs 2 and 3 are co-transcribed. Downstream of the ORFs 1 and 3 we found regions of extended dyad symmetry, which may probably function as transcription termination signals.

The sequence of the fragment V735 was determined and compared to the sequence of the fragment V10. No major deletions or insertions were found between the sequenced regions. The ORFs 1, 3 and 4 of strains 10 and S735 were highly homologous. The putative protein fragments encoded by the ORFs 1 differed in 2 (1,3%) amino acids; the putative proteins encoded by the ORFs 3 differed in 19 (4.5%) amino acids (Fig. 4B), whereas the putative protein fragments of the ORFs 4 were identical. However, major differences were observed between the ORFs 2 of strains 10 and S735. In the pathogenic strain 10 an ORF of 699 bases was found, predicting a protein product of 233 amino acids. In contrast, due to a frame-shift mutation, in the weak-pathogenic strain S735 an ORF of 569 bases was found, coding for a polypeptide of 183 amino acids. Compared to the putative protein encoded by strain 10, the putative protein encoded by strain S735 lacked the N-terminal 50 amino acids (Fig. 4A). Beside these N-terminal differences, the putative proteins differed at 9 amino acid positions (4.9%). In addition, the putative -35 regions that may be part of the promoter sequences, involved in the expression of ORFs 2 and 3, differed between the two strains. A TGGACA sequence was found in strain 10, whereas a TGGTCA

sequence was found in strain S735. The sequence data suggest that the differences in the virulence-enhancing effects of the fragments V10 and V735 may be the results of functional differences between the putative proteins expressed by the ORFs 2 and/or 3, and/or by differences in their levels of expression.

5   ORF 2 or ORF 3?

To examine whether the observed increase of the fragment V10 on virulence resulted from ORF 2 or ORF 3 or both, we next constructed the plasmids pCOM-V10-ORF2 and pCOM-V10-ORF3, containing the individual ORF 2 and ORF 3 encoding regions. Because ORF 3 most probably is co-transcibed with ORF 2, in pCOM-V10-

10   ORF3 the ORF 3 encoding region was preceded by the promoter region of ORF 2. Subsequently we determined the virulence properties of the strains S735(pCOM-V10), S735(pCOM-V10-ORF2), S735(pCOM-V10-ORF3) and S735(pCOM1). As shown in Table 2E the fragements V10 and ORF 2 showed similar effects on the virulence of strain S735. No effect of ORF 3 on the virulence of strain S735 could be observed.

15   These data show that ORF 2 is responsible for the observed effect on virulence and that the ORF 2 protein is an important virulence factor.

Distribution of the orf2 and orf3 sequences among all known 35 S. suis serotypes. To examine the homology between the orf2 and orf3 genes and genes of other S. suis serotypes, we performed cross-hybridization experiments. DNA

20   fragments of the orf2 and 3 genes were amplified by PCR, labelled by ^{32}P, and hybridized to chromosomal DNAs of the reference strains of the 35 different S. suis serotypes. As a positive control we used a probe specific for 16S rRNA (24). The 16S rRNA probe hybridized with almost equal intensities with all serotypes tested (results not shown). Probes orf2 and orf3 hybridized with all serotypes, except for

25   serotypes 32 and 34 (results not shown). This indicates that the proteins encoded by orf2 and 3 are common among most Streptococcus species

Herein we thus provide the development and the successful application of an in vivo complementation approach for the identification of important molecular determinants that determine the differences in virulence between pathogenic and

30   weak-pathogenic strains of Streptococcus. Using the complementation approach one unique clone, containing a 3.0-kb fragment of pathogenic strain (V10), was selected. The selected fragment was greatly enriched in seven different pigs and the observed enrichment was not tissue specific. The selected fragment showed similar enhancing

effects on the virulence of two different weak-pathogenic strains. Large differences were observed between the effects of the selected fragment V10 of the pathogenic strain 10 and the corresponding fragment V735 isolated from the weak-pathogenic strain S735 on virulence. In contrast to V10, which had a strong virulence-enhancing

5   effect on weak-pathogenic strains, V735 showed only minor effects. Therefore, differences between these two fragments are considered responsible for the observed differences on virulence. Sequence data showed that the fragments V10 and V735 were highly homologous. Both fragments contained two complete ORFs (ORFs 2 and 3), both of which can potentially express proteins that may further contribute to the

10 observed effect on virulence. The ORFs 3 are highly homologous and differ in only 19 amino acids. The proteins encoded by the ORFs 3 showed homology to FolC (folylpolyglutamate synthetase) of various pro- and eucaryotic organisms. Folylpolyglutamate synthetase catalyzes the conversion of folates to polyglutamate derivatives (4). Bacteria require folates for the biosynthesis of glycin, methionine,

15 formylmethionine, thymidine, purines and patothenate (4). Whether the FolC proteins encoded by the fragments V10 and V735 have different enzymatic activities or different substrate specificities is unknown so far. In E. coli a folC mutant is methionine deficient (4), however, so far a role of FolC in virulence has not been described. Significant differences were also observed between the ORFs 2 of the

20 fragments V10 and V735. Compared to the putative ORF 2 protein encoded by strain 10, the putative protein encoded by strain S735 lacked the N-terminal 50 amino acids. In strain S735 a strong ribosome-binding site precedes the methionine startcodon of ORF 2. In contrast, however, in strain 10 the sequence did not indicate the presence of a strong ribosome-binding site preceding the methionine startcodon of

25 ORF 2. Therefore, although ORF 2 of strain 10 is extended compared to ORF 2 of strain S735, it is not clear whether the proteins expressed by these two ORFs differ in length. Future experiments will be required to analyze the expressed proteins in detail. In addition to the putative N-terminal differences, the putative ORF 2 proteins differed at 9 amino acid positions (4.9%). Except for one, these amino acid

30 substitutions were clustered at two different positions in the putative protein. The function of the ORF 2 protein is unknown so far. Not even distant or partial homologies were found between the ORF 2 protein sequences and protein sequences present in the data libraries. Hydrophobicity profiles showed that the ORF 2 encoded

protein(s) is very hydrophobic. A role of the ORF 2 protein in the cellular membrane is therefore suggested. The putative -35 region preceding the ORFs 2 and 3 differed between strains S735 and 10. Therefore, we cannot exclude that differences in the expression levels rather than functional differences are responsible for the observed

5   effects on virulence.

In previous experiments we found that pigs infected with weak-pathogenic strains showed only mild clinical signs of disease and that bacteria could never be reisolated from the CNS or the joints (33, 34). Surprisingly, in the experiments described in this paper, in which we used weak-pathogenic strains containing the

10   control plasmid pCOM1, bacteria could (with a low frequency) be re-isolated from the CNS as well as from the joints. There are several possible explanations for these observed differences. One explanation is that the presence of the plasmid somehow affects the (virulence) properties of the strains. Another possibility is that the treatment of the pigs with erythromycin makes the pigs more sensitive for *S. suis*

15   infections and a third possibility is that compared to the pigs used previously, the pigs used for the current experiments were more sensitive for *S. suis* infections.

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Table 1. Bacterial strains and plasmids

strain/plasmid	relevant characteristics*	source/reference
Strain		
E. coli		
XL2 blue		Stratagene
S. suis		
10	pathogenic serotype 2 strain	(33)
S735	weak-pathogenic serotype 2 reference strain	(34)
24	weak-pathogenic serotype 2 strain	(33)
Plasmid		
pKUN19	replication functions pUC, Amp^R	(14)
pE194	Em^R	(11)
pMR11	pKUN19 containing *S. suis* mrp gene	(25)
pCOM1	replication functions pWVO1, Em^R	this work
pCOM-L	pCOM1 containing random sequences of *S. suis* strain 10	this work
pCOM-V10	pCOM1 containing *S. suis* strain 10 fragment selected in pigs	this work
pCOM-V735	pCOM1 containing a 3.1 kb *PstI-Hind*III fragment from *S. suis* strain S735 (homologous to V10)	this work

*

Spc^R: spectinomycin resistant

Amp^R: ampicillin resistant

Em^R: erythromycin resistant

Table 2. Virulence of *S. suis* library and strains in germfree pigs.

strains	no. of pigs	dose	mortality[a] (%)	mean no. of days till death	morbidity[b] (%)	clinical index of the group		fever index[e]	leucocyte index[f]	no. of pigs in which S. suis was isolated from		
						specific[c] symptoms	non-specific[d] symptoms			CNS	serosa	joints
A												
S735(pCOM-L)	4	10^7	100	4	100	69	91	25	n.a	3	2	3
S735(pCOM-L)	4	10^8	75	7	100	50	69	20	17	2	1	2
B												
S735(pCOM-V10)	5	10^6	100	1	100	100	100	54	4	5	5	5
S735(pCOM1)	4	10^5	25	12	25	2	11	6	80	1	1	2
C												
S735(pCOM-V10)	5	10^6	100	1	100	100	100	60	n.a	5	5	5
S735(pCOM-V735)	5	10^6	20	15	100	40	26	17	52	1	1	1
S735(pCOM1)	5	10^6	20	16	60	11	9	11	20	1	0	0
D												
24(pCOM-V10)	5	10^6	100	2	100	50	66	42	29	3	3	5
24(pCOM-V735)	4	10^6	25	15	100	40	30	17	18	1	0	0
24(pCOM1)	5	10^6	20	15	20	2	14	6	21	1	0	0
E												
S735(pCOM-V10)	4	10^6	100	1	100	100	100	57	n.d.	4	4	4
S735(pCOM-V10-ORF2)	4	10^6	100	1	100	100	84	50	n.d.	4	4	4
S735(pCOM-V10-ORF3)	4	10^6	0	11	0	6	4	3	n.d.	0	0	0
S735(pCOM1)	4	10^6	0	11	0	0	9	5	n.d.	0	0	0

[a] Percentage of pigs that died due to infection or had to be killed for animal welfare reasons
[b] Percentage of pigs with specific symptoms

21

[c] Percentage of observations for the experimental group in which specific symptoms (ataxia, lameness of a least one joint and/or stillness) were observed

[d] Percentage of observations for the experimental group in which non-specific symptoms (inappetite and/or depression) were observed

[e] Percentage of observations for the experimental group of a body temperature of > 40° C

[f] Percentage of blood samples for the experimental group in which the concentration of granulocytes was > 10^{10}/liter

n.a.: not applicable

n.d.: not determined

LEGEND TO FIGURES

Fig. 1.

5   The pCOM1 vector used in this study. pCOM1 contains the replication functions of

pWVO1 (13), the erythromycin-resistance gene of pE194 (11) preceded by the

promoter region of the *mrp* gene (25) as well as the *SacI-PstI* part of the multiple

cloning site of pKUN19 (14).

10   Fig. 2.

Plasmids digested with *SmaI* and *XbaI* on an 0.8% agarose gel. Library: plasmids

isolated from 10 randomly selected clones of the original library; clones enriched in

pigs: plasmids isolated from 19 independently selected clones enriched in pigs; c:

pCOM1; m: molecular size marker.

15

Fig. 3.

Schematic representation of the fragments V10 and V735. The arrows indicate the

potential ORFs. æ P, indicates the position of the potential promoter sequence; °,

indicate the positions of the potential transcription regulator sequences. Homologies

20   (% identities) between the potential proteins encoded by the ORFs and proteins

present in the data libraries are indicated.

Fig. 4. A, B

Homology between the ORFs2 (A) and 3 (B) encoding proteins of fragments V10 and

25   V735. The asterisks indicate the non-identical amino acids.

Fig. 5. A, B, C, D, E, F

Nucleotide and amino acid sequences of fragments of V10 and V735.

CLAIMS

1.   A method for modulating virulence of a *Streptococcus* comprising modifying a genomic fragment of said *Streptococcus* wherein said genomic fragment comprises at least a functional part of a fragment identifiable in *Streptococcus suis* as a nucleic acid or fragment thereof as shown in figure 5.

5   2.   A method for assaying virulence of a *Streptococcus* comprising assaying a genomic fragment of said *Streptococcus* wherein said genomic fragment comprises at least a functional part of a fragment identifiable in *Streptococcus suis* as a nucleic acid or fragment thereof as shown in figure 5.

3.   An isolated and/or recombinant nucleic acid derivable from *Streptococcus* and
10 identifiable in *Streptococcus suis* as a nucleic acid or fragment thereof as shown in figure 5.

4.   A vector comprising a nucleic acid according to claim 3

5.   A host cell comprising a nucleic acid according to claim 3 or a vector according to claim 4.

15   6.   A host cell according to claim 5 comprising a *Streptococcus*.

7.   A vaccine comprising a nucleic acid according to claim 3 or a vector according to claim 4 or a host cell according to claim 5 or 6.

8.   A protein or fragment thereof encoded by a nucleic acid according to claim 3.

9.   An antibody directed against a protein or fragment thereof according to claim 8.

20 10.   An antigen comprising a protein or fragment thereof according to claim 8

11.   A diagnostic test comprising an antibody according to claim 9.

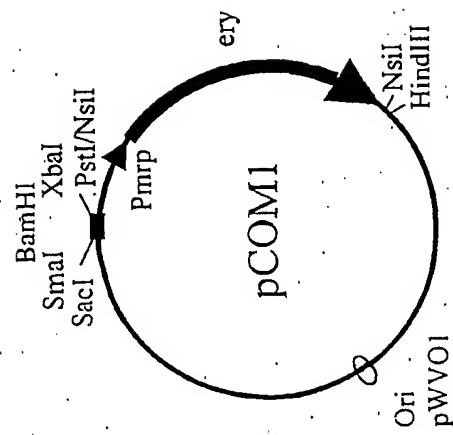12.   A vaccine or diagnostic test comprising an antigen according to claim 10.
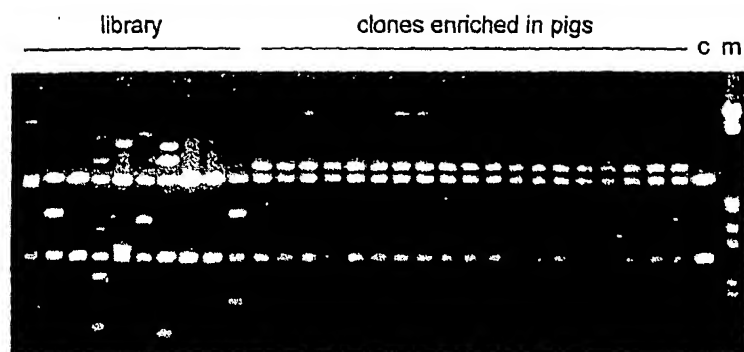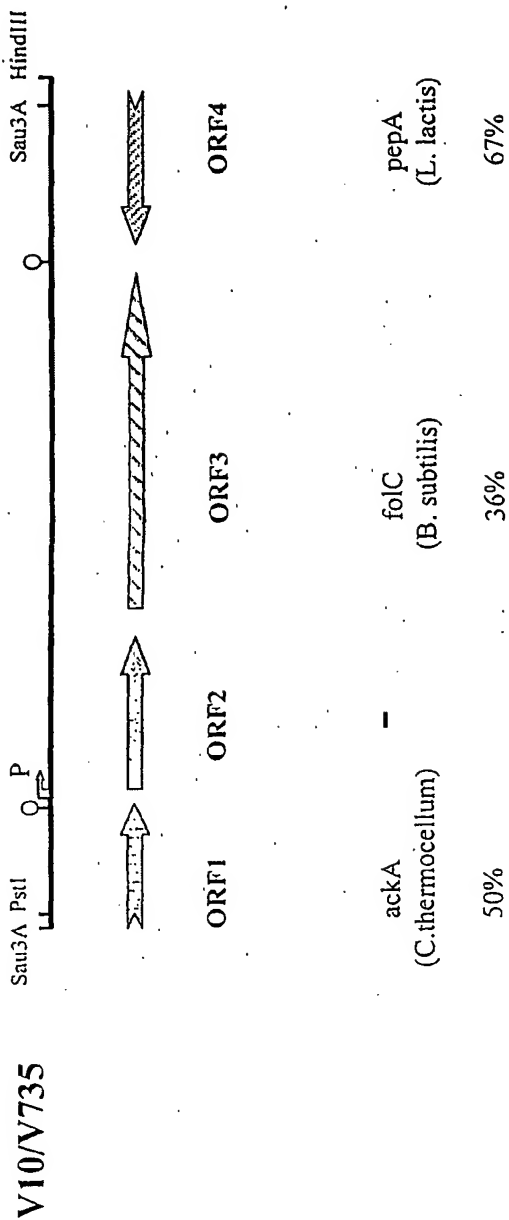
FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4A

A

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                    ORF2 V10
                    ORF2 V735

  1   MLPHNEADLCLHIMSPRVGTLVLAESSAVNHCIRCRIHTAPFFEKGAFFMEKKIPKLIV
                                                    MEKKIPKLIV

 61   QLLAAIAMTLALVMIVENYFSIRISDILQVQFTFTPNTILGAIAGPWAAVFAAISDPVF
                     * * * *                                   *
      QLLAAIAMTLALVMIAENHFSVRLSDILQVQFTFTPNTILGAIAGPWAAVFAAISDPAF

121   VLFSGQIVLFTWILLEAVSAFTYGWFFYRKPLDTKNKADMLYVAGVVLIQVVISFIMTP
           ** **
      VLFSGQSMLFSFTLLEAVSAFTYGWFFYRKPLDTKNKADMLYVAGVVLIQVVISFIMTP

181   IALHFHFGTPWIVLYSSRLIKAVEEIPLRIVVIMIVLPSLQKIPELAKLMGIK
      IALHFHFGTPWIVLYSSRLIKAVEEIPLRIVVIMIVLPSLQKIPELAKLMGIK
```

FIGURE 4B

ORF3 V10
ORF3 V735

B

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  1    MNYQETRRWLSSRPASDLENGVARVNWILERLDNPQLQVPTVHFVGINGKGSTINALQSI
       MNYQETRRWLSSRPASDLENGVARVNWILERLDNPQLQVPTVHFVGINGKGSTINALQSI

 61    LQSSDYTVGRFTSPSIIDEREQIVQQEMISEEDFARIVTDLQPLIEDLDQTAGLDAISE
          *                  **  **
       LRSSDYTVGRFTSPSIIDEREQIVEEQFMISEEDFARIVTDLQPLIEDLDQTAGLDAISE

121    FEIVVVAMFVYFAHYQRPDILLVEAGVGGLQDATNVLAPLAVVCPSIGLDHQAFLGETHA
       FEIVVVAMFVYFAHYQRPDILLVEAGVGGLQDATNVLAPLAVVCPSIGLDHQAFLGETHA

181    ALARHKVAVLREGVPLLYATDQPEVETVEEHACQLQSPTYAVGREILLENSRAGEAVSS
                 *    *    **** *  * *
       ALARHKVAVLRERVPLLYATDQSEVVAAFEDHASQLQSPTYAVGREILLENSRAGEAVSS

241    PLGRVEELTLQMQGRHQEVNAALAVTTAQLIKPHFPTINETIRQGLSQAIWPGRLELIR
        *                         *****
       TLGRVEELTLQMQGRHQEVNAALAVTTAQLLSPDEPTINETIRQGLSQAIWPGRLELIR

301    PNIMIDGAHNNESIAVLIQLLEEKYADRDIEILFAAINTKPVDQMLSQLSQFGPVSVTTF
       PNIMIDGAHNNESIAVLIQLLEEKYADRDIEILFAAINTKPVDQMLSQLSQFGPVSVTTF

361    DDFRAVQLEDYPSGYERVQITYQEWVEQADLDNPKKLYLITGSLYFTTYVRKYILEELV
               *                 *  *    *
       DDFRAVQLGDYPSGYERVQITYQEWLEQVDLDNPKQLYLITGSLYFTTYVRKYILEELV
```

FIGURE 5A V10 seq.

GGATCCTGCTATCATTCCTTATTTGATTGCGAATGTTGAAGAACTGAAAGATGCTGCAGACGTTGTTAACATGTTGAATA
AACAGTCAGGCTTATTCGGTGTATCTGGCTTCTCAAGTGATATGCCGTGATATTGAAGCAGGTATCCAAGCTCACAATCCA
GATGCAGTGTTGGCCTACAATATTTCATTGACCGTATTAAGAAATTTATCGGTCGCCAATGACGTAGTAGAAGGTTTGTCTTGGT
AGATGCTATTGTCTTCACCGGCTGGTATGGGTGAAAATGCACCCGTTATGCGGCAATGACGTAGTAGAAGGTTTGTCTTGGT
TTGGTATTGAGTTGGACCTACAAAAAATGTATTCGGCAACTATGGTGACATTTCAACGGCAGAATCAAAAGTTCGTGTC
TTGGTTATTCGACGGATGAAGAATTGGTTATTGCGCGTGAAGTGGAACGCTTGAAATAAGAAAACTAACTGGTAGTCG
GAGACTGCCAGTTTCTCTTATAGTTTATACCTTTAGAAAGGTATAGTTTTTAGCAAGTGGACAAAATATATAGTGTGTGA
TACAATAGACTAGCAAAGAAATTGCACAGAGTAGATGGTTTGCGTCAAGTGTATGTGGATGGGATGTTGCCACATAAACG
AAGCTGATCTTGCTTGCCATCTGATGTCTCCTAGAGTAGGAACATTGGTCCTGGCTGAGAGTAGCGCGGTAAACCATTGC
ATCCGCGTGAATACACGACCAGCTCCATTTTTGAAAAAGGAGCATTTTATGGAAAAGAAAAATTCCAAAACTAAC
GGTGCAGTTGTTGGCTGCTATTGCGATGACCCTTGCCTTGGTCATGATTGTAGAGAACTATTTCTATTCGGATTTCTG
ATACTTTACAGGTTCAGTTACCTTCATTCCAATACTATTTGGGAGCTATTGCGCGGTCCCTCTTCACTTGGATTTGATTGAGGCGGTATC
GCGGCTATTTCAGACCCAGTCTTGTCTTGTTAGCGGGCAAACGGTCCTCTTCACTTGGATTTGATTGAGGCGGTATC
GGCATTTATCTTACACGGCTGGTTCTTCTATCGAAAACCGCTAGACACCAAGAACAGGCTGATTGGCTCTATGTGGCTGGTG
TAGTTGTCTTGATTCAGGTTGTGATTCCTTTATCATGACACCGATTGCCCTCCATTTCATTTGGAACACCTTGOATT
GTTCTGTATAGCAGTCGCTTGATTAAGGCAGTTTTTGAAATTCCATTACGGCATTGTCGTGACCATGCTTGTCTTGCCAAG
TTTACAAAAATACCTGAATTGGCCAAGTTAATGGCCATTAAATAAAACAGTATCAAGCAACAGGTCATCCCCTGTTGC
TACTTTTGTAGAGAGGGAATCATGAATTATCAAGAAACTCGCCGGTGGCTATCTAGTCGTCCTGCATCAGATTTAGAAAA
TGGCGTTGCACGTGTCAACTGGATTTTAGAACGCTTGGACAATCCCAGCTTCAAGTGCCGACCGTACACTTGTCGGCA
CAAATGGCAAGGGCTCGACCCTCAACGCTTCACAGTCTCATCTTCGGATTACACCGTCGGCCGCCTTTACATCA
CCGTCTATCATTGATTTCGAGAGCAGATTGTCTACCAGCAGGAGATGATTTCGGAGGAAGATTTTCGGAGGATTGTGAC
AGACTTGCAACCCTTGATCGAGGACTTGGACCAGACGGCGTCATCTCGGAGGTTTGAGATTGTAGTAGTGG
CTATGTTGTCTACTTTGCCCCACTACCAGCCTCCCGATATTCTCTTGGTGGAGGCCGGCATGGGTGGTTGCAGGATGCG
ACCAATGTCCTTGCCTCCCTTGGCAGTAGTTGTCCGTCCATCGGTTTCGTCCATCGGTTTGGACCATCAGGCATTTTGGGAGAGACCCACGC
TGCTATAGCCCGTCACAAGGTCGCCGTCTTGCGGTGAGGGGGTCCGCCTCATCTATGCGACCATGGGCGGGAGAAGTGGAGA
CAGTATTTGAGGAGCATGCCTGTCGTCAGCTTCAGATCCGACCTATGCGGTGGGCGGGAGATTCTTTTTGAAAAATAGCAGA
GCAGGCTTTGCAGTTTCAAGTCCTCTCGGCCGTGTGGAAGAGTTAACACTACAGATGCAGGGTCGTCACCAGAGGTCAA
TGCAGCCTTGGCAGTGACAACAGCTCAGCTCACTCATTAAACCTCATTTTCCAACAATTACCAATGAAACCATCCGCCAGGGCT

FIGURE 5A V10 seq. , Contd.

```
TGTCCCAAGCCATCTGGCCGGGTCGCTTAGAGTTGATTAGGCCTAATCTCATGATTGACGGTGCCCACAATAATGAAAGT

ATCGCCGTCCTGACACAACTCTTGGAAGAAAAGTATGCTGACAGGGATATTGAAATCCTCTTTGCGGCCATCAATACCAA

GCCAGTGGACCAGATGTTGTCCCAGCTTAGCCAATTGGACCTGTTAGCGTGACGACCTTTGACGATTTCAGAGCGGTAC

AGTTAGAAGAGATTATCCGTCAGGCTATGAACGAGTTCAGACCTATCAGGAGTGGGTGGAGCAGGCGGACTTGGACAATCCC

AAAAACTCTACCTGATTACAGGCTCGCTATATTTCATTACCTATGTGAGGAAGTACATTTTAGAAGAACTTGTTTAGAA

AAAAAGGCTTTGCCGGGCATTCAACCCAGCAAAGTCTTTGTTTAATAATTTTAATCAAATCAACCGTTGAGCGGTC

TAGTTTTTAACGATGGTCTGCAAGAAGGCTTGGGCCTCTAAGAAGTCATCCATGCTGTAGAGAGTTTGATGTGAATGGA

TGTAGCGGAGCGCAGACACCGATAGTTGTTGATGAACACCATGATTTTCAAGTGGGCTGCACCAGCATCTGTTCCACCT

TTACCACAGTAGTATTTGACACCTGCTTCTTCGGCAGTTGTGAGGAGGAAGTCTTTCATGTTTTTTAACATGAT

GTGACCTGGATCC
```

FIGURE 5B V735 seq.

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CTGCAGATGTTGTGAACATGTTGAATAAACAGTCAGGCTTGTTCGGTGTATCTGGCTTCTCAAGTGATATGCGTGATATT
GAAGCAGGCATCCAAGCTCACAATCCAGATGCAGTGTTGGCCTACACAATATTTCATTGACCGTATTAAGAAAATTTATCGG
TCAGTATCTTGCAGTTTAAATGGGGCAGATGCTATTGTCTTCACGGCTGGGTGATGGGTGAAAAATGCACCGCTTATGCGCA
ATGACGTAGTAGAAGGCTTGTCTTGGTTGGTATTGAGTTGGACCCACAAAAAAATGTATTTGGCAACTATGGTGACATT
TCAACGGCAGAATCAAGGGTTCGTGTCTTGGTTATCCGACGGATGAAGAAGTTGGTTATTGCGCGTGAAGTTGAACGTTT
GAAATAAGAAAAACTAACTGGTAGTCGGAGACTGCCGGTTTCTTCTTATAGTTTATACCTTTAGAAAGGTATAGTTTTTAG
CAAGTGGTCAAAATATATAGTGTGATACAATAGACTAGCAAAGAAATTTGCACAGAGTAGATGGTTTGCGTCAAGTGT
ATGTGGATGGGATGTTGCCACATAACGAAGCTGATCTTTGCTTGCCATCTGATGTCTCCTAGAGTAGGAACATTGGATCTG
GCTGAGAGTAGCGCGGTAAACCATTGCATCCGCGTGTCGAATACACACGACAGCTCCATTTTTTGAAAAGGAGCATTTTT
ATGGAAAAGAAGATTCCAAAACTAACGGTGCAGTTGTTGGCGATGGTCCTGTCGGATGACTCTTGCCTTGGTCATGATTGCGGA
GAACCATTTTCTGTTCGTCTTCTGATACCTTGCCAGTTTACCTTTATCCCTAATACTATTTTAGGTGCGATTG
CTGGTCCTGTTGGGCGTCGTGTATTTGCGGGCGTATCGGCTTTTATCTATGGCTGGTTCTTCTCATCGAAAAACCGCTAGACACCAAGAACAA
AGTTTATCTTGATTGAGGCGGGTATCGGCGGGGTTGTTGTTCTTCAGGTGTTGATTTCGATTCCTTTATCATGACCGATTGCCCTCC
GGCTGATTGGCTCTATGTGCAGGGGTGTTGTTGTCTGTATAGCAGTGCGTTGATTAAGGCCGGTTTTTGAAATTCCATTACGGCATT
GTCGTGACTATGCTGTCTTACCAAGTTTACAAAAAATACCTGAAGTTAATGGCATTAAAACTCGCCGGTGGCTATCT
CAAGCAACAGGTCATCCCCCGTGTTGCTCGTTTTGTAGAGAGGAATCATGAAGAACTGGAATCATCAAGAAACTCGCCGGTGGCTATCT
AGTCGTCCTGCATCAGATTTAGAAAAATGGCGTTGCACGTGTCAACTGGATTTTGGAACGCTTGGACAATCCCCAGCTTCA
AGTGCCGACCGTTCACTTCGTAGGTACAAAATGGCAAGGGCTCGACCCTCAACGCCTTACAGTCTATCTTACGGTCTTCGG
ATTACACCGTCGGTCGGTCGCTTTACCTCACCGTCTATCATGATTTTCAACCCTGATTGAGGACTGGACCAGCGTCCCGACATTCTCTGTGTGGAGG
GAGGAAGATTTTGCAAGGATTGTGACAGACTTGCAACCCTGATTGAGGACTGGACCAGCGTCCCGACATTCTCTGTGTGGAGG
CTCGGAGTTTGAGATTGTGAGTAGTGGCTATGTTTGTCTACTTTGCCCCATTGGCCAGTAGTTGCCCGTCCATCGGCTTGGACCAT
CGGGCATGGTGGTTTGCAGGATGGCGACCAATGTCCTTGCCCATAGCCCGTCTATAGCCGTCTATAGCGGTTCCCCTCCTA
CAGGCTTTTTTGGGGAGAGACCCACGCTGCTATAGCCCGTCTACAAGGTTGCTGTCTTGCGTGAGCGGGTTCCCCTCCTA
TGCGACCGACCAGTCAGAAGTGGTGGCAGCATTTGAGGATCACGCCCAGCTTCAGAGTCCGACCTATGCGGTGGGAC
GGGAGATTCTTTTGGAAAATAGCAGAGGAGGCTTTGTGTTTCAAGTACCTCTCGGCCGTGTGAAGAATTAACACTGCAG
ATGCAGGGTCGTCACCGGAGGTCAATGCAGCCTTGGCAGTGACAACAGCTCAGCTTCTCAGCCCTGATTTTCCAACAAT
TACCAATGAAACCATCCGCCAGGGCTTGTCCCAAGCCATCTGGCCGGGCCGCTTAGAGTTGATTAGGCCTAATCTCATGA
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FIGURE 5B V735 seq., Contd.

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TTGACGGTGCCCACAATAATGAAAGTATCGCCGTCCTGACACAACTCTTGGAAGAAAAGTATGCTGACAGGGATATTGAA
ATCCTCTTTGCGGCCATCAATACCAAGCCAGTGGACCAGATGTTGTCCCAGCTTAGCCAATTTGGACCTGTTAGCGTGAC
GACCTTTGACGATTTCAGAGCGGTACAGTTAGGAGATTATCCGTCAGGCTATGAACGAGTTCAGACCTATCAGGAGTGGT
TGGAGCAGGTGGACTTGGACAATCCCAAACAACTCTACCTGATTACAGGCTCGCTATATTTCATTACCTATGTGAGGAAG
TACATTTTAGAAGAACTTGTATAGAAAAAAGGCTTTGCCGGGCATTCAACCCAGCAAAGTCTTTGTTTTAATAATTTTT
AATCAAATCAACCGTTGAGCGGTCTAGTTTTTAACGATGGTCTGCAAGAAGGCTTGGGCCTCTAAGAAGTCATCCATGC
TGTAGAGAGTTTGATGTGAATGGATGTAGCGAGCGCAGACACCGATAGTTGTTGATGGAACACCATGGTTTTTCAAGTGG
GCTGCACCGGCATCTGTTCCACCTTTACCACAGTAGTATTGGAATTTGACACCTGCTTCTTCGGCAGTTGTGAGGAGGAA
GTCTTTCATGTTTTTAGCATGATGTGGCCTGGGTCATAGAAACGAAGCAGAGTTCCGTCACCAATTTTTCCTTGGTCGC
CATAAATATCACCTGCGGGGCGAGCAATCAACAGCGAGGAGAAAATGTCTGGATTGAACTTGGTTGTTGTAGAGGCATGAGCACCA
CGAAGACCAACCTCTTACTTGCACATTGGCCCCAGCAATCAACTGATTTGCAAAGCTT
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FIGURE 5C ORF 2 V10

MLPHNEADLCLHLMSPRVGTLVLAESSAVNHCIRCRIHTIAPFFEKGAFFMEKKIPKLTVQLLAAIAMTLALVMIVENYF
SIRISDTLQVQFTFIPNTILGAIAGPVWAAVFAAISDPVFVLFSGQTVLFTWILIEAVSAFIYGWFFYRKPLDTKNKADW
LYVAGVVVLIQVVISFIMTPIALHFHFGTPWIVLYSSRLIKAVFEIPLRIVVTMLVLPSLQKIPELAKLMGIK

FIGURE 5D ORF 2 V735

MEKKIPKLTVQLLAAIAMTLALVMIAENHFSVRLSDTLQVQFTFIPNTILGAIAGPVWAAVFAAAISDPAFVLFSGQSMLF
SFILIEAVSAFIYGWFFYRKPLDTKNKADWLYVAGVVVLIQVVISFIMTPIALHFHFGTPWIVLYSSRLIKAVFEIPLRI
VVTMLVLPSLQKIPELAKLMGIK

FIGURE 5E ORF 3 V10

MNYQETRRWLSSRPASDLENGVARVNWILERLDNPQLQVPTVHFVGTNGKGSTLNALQSILQSSDYTVGRFTSPSIIDFR
EQIVYQQEMISEEDFARIVTDLQPLIEDLDQTAGLDAISEFEIVVAMFVYFAHYQRPDILVEAGMGGLQDATNVLAPL
AVVCPSIGLDHQAFLGETHAAIARHKVAVLREGVPLIYATDQPEVETVFEEHACQLQSPTYAVGREILLENSRAGFAVSS
PLGRVEELTLQMQGRHQEVNAALAVTTAQLIKPHFPTTNETIRQGLSQAIWPGRLELIRPNLMIDGAHNNESIAVLTQL
LEEKYADRDIEILFAAINTKPVDQMLSQLSQFGPVSVTTFDDFRAVQLEDYPSGYERVQTYQEWVEQADLDNPKKLYLIT
GSLYFITYVRKYILEELV

FIGURE 5F ORF 3 V735

MNYQETRRWLSSRPASDLENGVARVNWILERLDNPQLQVPTVHFVGTNGKGSTLNALQSILRSSDYTVGRFTSPSIIDFR

EQIVFEQEMISEEDFARIVTDLQPLIEDLDQTAGLDAISEFEIVVAMFVYFAHYQRPDILLVEAGMGGLQDATNVLAPL

AVVCPSIGLDHQAFLGETHAAIARHKVAVLRERVPLLYATDQSEVVAAFEDHASQLQSPTYAVGREILLENSRAGFAVSS

TLGRVEELTLQMQGRHQEVNAALAVTTAQLLSPDFPTITNETIRQGLSQAIWPGRLELIRPNLMIDGAHNNESIAVLTQL

LEEKYADRDIEILFAAINTKPVDQMLSQLSQFGPVSVTTFDDFRAVQLGDYPSGYERVQTYQEWLEQVDLDNPKQLYLIT

GSLYFITYVRKYILEELV